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Epigenetic control of prolyl and asparaginyl hydroxylases in prostate cancer

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14. ABSTRACT
In many solid tumors, including prostate cancer, hypoxia-inducible factors (HIF) are up-regulated compared to their normal tissue counterparts. These HIF molecules are transcription factors, and supply the cancer with the proper proteins necessary for metabolism as well as angiogenesis. Recently, a set of proteins known as prolyl and asparaginyl hydroxylases (PHD and AHD respectively) have been shown to be essential in the regulation of HIF, and in some cancers have been transcriptionally and translationally silenced. We therefore proposed a study that focuses on the epigenetic control of these crucial enzymes. In this report, we present data demonstrating our first findings of PHD expression in prostate cancer cell lines as well as expanding our studies to relevant human samples. Furthermore, we begin to identify specific epigenetic mechanisms that may play a major role in the transcriptional and translational control of these enzymes. Last, we will explain our future direction of the project for the remainder of the award period.

15. SUBJECT TERMS
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Introduction

In many solid tumors, including prostate cancer, hypoxia-inducible factors (HIF) are up-regulated compared to their normal tissue counterparts. These HIF molecules are transcription factors, and supply the cancer with the proper proteins necessary for metabolism as well as angiogenesis. Recently, a set of proteins known as prolyl and asparaginyl hydroxylases (PHD and AHD respectively) have been shown to be essential in the regulation of HIF, and in some cancers have been transcriptionally and translationally silenced. We therefore proposed a study that focuses on the epigenetic control of these crucial enzymes. In this report, we present data demonstrating our first findings of PHD expression in prostate cancer cell lines as well as expanding our studies to relevant human samples. Furthermore, we begin to identify specific epigenetic mechanisms that may play a major role in the transcriptional and translational control of these enzymes. Last, we will explain our future direction of the project for the remainder of the award period.

Body

In our previous report, we had begun the initial characterization of the PHD's and AHD in an array of prostate and breast cancer cell lines. In contrast with our initial hypothesis, the expression levels of PHD1, PHD2, and AHD did not significantly differ amongst the cancer cell lines compared to normal controls. On the contrary, the mRNA and protein level of PHD3 was quite variable amongst the cell lines assayed. Due to this observation, our initial statement of work was changed to focus on this specific isozyme. We attempted to examine the role of epigenetic silencing of PHD3 in these cell lines, and found that DNA methylation appeared to be a key regulator of the expression level of this protein. Furthermore, it was found that PHD3 could be reactivated if given a potent DNA de-methylating agent (5-aza-dC) demonstrating a causal relationship to the methylation of the PHD3 promoter and gene silencing. The epigenetic silencing of PHD3 was not limited to DNA methylation, as it was observed that global chromatin structure was also altered around the PHD3 promoter. At the time of the last report, we had only preliminary evidence of this, but repeating these experiments demonstrated this trend to be a significant finding in the prostate cell line PC3 (Figure 1). While these results are novel and exciting, using only prostate carcinoma cell lines leaves questions about clinical

relevance of our findings. To address this issue, we have begun characterization studies upon prostate tumor samples extracted from human patients. Due to the recent acquisition of these samples, our preliminary examination demonstrated that the majority of prostate tumors expressed less PHD3 mRNA than the normal controls (Figure 2). We intend on extending this finding to the protein level (e.g. Western blots) as well as examining the PHD3 promoter methylation status in these samples as well. The use of these samples should add a clinically relevant corollary to this project that we feel is critical for prostate cancer patients.

Our studies have shown that DNA methylation appears to be a main regulator of gene control in prostate and breast cell lines. To expand our current data looking solely at the PHD3 promoter, we have used a new technology known as "MethylMiner." Briefly, by using recombinant methylated DNA binding proteins, we are able to separate entire genomic fractions based on the presence or absence of methylation. Once this is complete, we are able to query the samples for any gene of interest to understand if the methylation is increased or decreased compared to another cell line. To assess if the technology worked, we used control unmethylated and methylated DNA (Figure 3). This data demonstrated that the assay worked very efficiently as the unmethylated DNA remained in the supernatant while the methylated control bound the beads and needed a high salt wash to become eluted. We then expanded this to some of our cell lines and PHD3 as the target gene. The results demonstrated that high PHD3 expressing cells were of low-methylation status, while low PHD3 expressing cells were high-methylation status (Figure 4). This data correlated directly with the promoter methylation analysis we had performed in our previous report, thus validating this method for assessing genomic DNA methylation status. We aim to use this technique to all of our cell lines as well as query other genes of interest in the cells as well as potentially assay human tumor samples as well.

As previously mentioned, the PHD enzymes determine the steady-state levels of the HIF transcription factors. With the knowledge that PHD3 is down-regulated in an array of prostate and breast cancer cell lines, we set out to examine the status of the HIF proteins in these cancer types. It has been well established that both the HIF and PHD enzymes are inducible by hypoxia, but it we discovered that in low PHD3 expressing cell lines that hypoxia could not induce PHD3 (Figure 5). To extend this finding, we performed western blot analysis on HIF-1α in these cell lines and discovered that the HIF response to hypoxia was not attenuated

(Figure 6). Furthermore, this induction of HIF-1α appeared to be functional as all cell lines were able to transactivate a hypoxia response element that was transfected into the cells (Figure 7). The presence of a normal HIF-1 response in these cells suggests that the other PHD molecules are acting to control the levels of HIF-1 during times of normoxia even though the PHD3 isoform is down-regulated. This redundancy amongst the PHD family may be a method of compensation in the event one PHD is lost or becomes non-functional. We hypothesize that the PHD enzymes may have different K_m for oxygen and as such their expression may be altered to adapt to differing oxygen tensions. This may be why numerous solid tumors alter their level of PHD3 compared to normal tissue, as the oxygen tension and distribution in a solid tumor varies greatly compared to that in normal tissue. We feel that pursuing this observation is outside the scope of the current project, but in future studies it may be shown that oxygen levels regulating PHD/HIF may be crucial in the elucidation of the evolutionary advantage of the epigenetic silencing of PHD3 in cancer.

Last, it has recently been observed in multiple myeloma cell lines that the NF-κB transcription factor pathway appears to be more transcriptionally active when PHD3 is silenced. To date, the reason for this has not been fully elucidated. It is speculated that PHD3 may interact with IκB and as such have a direct role on the NF-κB pathway or may possess even non-hydroxylase mediated downstream effects. Future plans for this project aim to examine if this observation holds true in prostate and breast cancer cell lines, as well as expand our findings of PHD3 silencing outside of only the HIF transcription factor pathway into other major signaling cascades, cellular maintenance, as well as metabolism. These and other intriguing questions can only be addressed in the context of another funded proposal, perhaps by a future student that inherits part or all of the project after my tenure as a graduate student. These avenues will represent new frontiers of investigation into the function, specificity, and molecular targets of PHD3 and how it plays a role at the interface between metabolism and epigenetics.

Key Research Accomplishments

- Identification of PHD3 mRNA expression human prostate tumors
- Confirmation of open and closed chromatin structure around the PHD3 locus in expressing and non-expressing cell lines respectively
- Introduction of a novel methylation analysis technology with identification and confirmation of methylation status of breast and cancer cell lines
- Examination of improper PHD3 induction upon exposure to hypoxia in methylated breast and prostate cancer cell lines
- Identification of proper HIF-1α protein induction in breast and prostate cancer cell lines
- Observation of proper HIF-1α transactivation ability in breast and prostate cancer cell lines

Reportable Outcomes

Peer-reviewed publications:

- Venkataraman S., Fitzgerald, M.P., Place, T.L., **Case, A.J**., Vorrink, S., Teoh, M.L.T., Oberley, L.W., Domann, F.E. (2010) *PHD3 promoter methylation is a mechanism for loss of PHD3 expression in breast and prostate cancers.* (In revision at *PLoS One*).
- **Case, A.J.** et al. (2010) *Aberrant T-cell development in SOD2 deficient thymocytes: A novel role for mitochondrial superoxide in negative selection in the adaptive immune system*. (In preparation for *Free Radical Biology and Medicine*).
- **Case, A.J.** et al (2010). *The conditional loss of SOD2 results in an erythropoietic protoporphyria-like phenotype*. (In preparation).

Abstracts:

- **Case, A.J.** et al. (2009). *Heightened Susceptibility to Influenza Mortality in Immunodeficient Mice Caused by a T-cell Specific Defect in SOD2*. Blood. 114 (22): 661.
- **Case, A.J**. et al. (2009). *Deficiency of MnSOD in Hematopoietic Stem Cells Causes a Sideroblastic Anemia-Like Phenotype.* Blood. 114 (22): 787.

- **Case, A.J**. et al. (2009). *Heightened Susceptibility to Influenza Mortality in Immunodeficient Mice Caused by a T-cell Specific Defect in SOD2*. Free Radical Biology and Medicine. 47 (1): S41.

Awards:

- **Case, A.J.**, Award for Clinical and Translational Medicine, The University of Iowa Carver College of Medicine Research Week, Iowa City, IA, 2010.

Conclusion

In summary, this project progressed as outlined in the revised statement of work as proposed in the last funding period. A newly revised statement of work is attached to this document following the appendices. Aim 1 has been added to contain relevant human prostate tumors which will add greatly to the clinical relevance of our project. Furthermore, the introduction of the new MethylMiner technology will significantly expand the scope of methylation analysis on these cells. We feel that in the final funding period new targets for gene silencing in prostate carcinoma will be discovered and open new projects that will warrant further elucidation. Aim 2 has remained unchanged, and essentially completed as scheduled. Finally, Aim 3 has progressed significantly and we have added the expanding our findings outside the realm of only the HIF transcription factor pathway. In doing so, we hope to understand the global ramifications of the silencing of the critical regulatory gene PHD3. Our studies have required us to make adaptive changes in our original plan so as to truly understand the mechanism underlying this epigenetic silencing, and much has been learned from our approaches. Finally, we hope that someday this project may progress to an *in vivo* model or even a clinical trial if the proper epigenetic targets discovered, understood, and possibly manipulated for a beneficial clinical outcome for patients.

Appendices – Figure 1

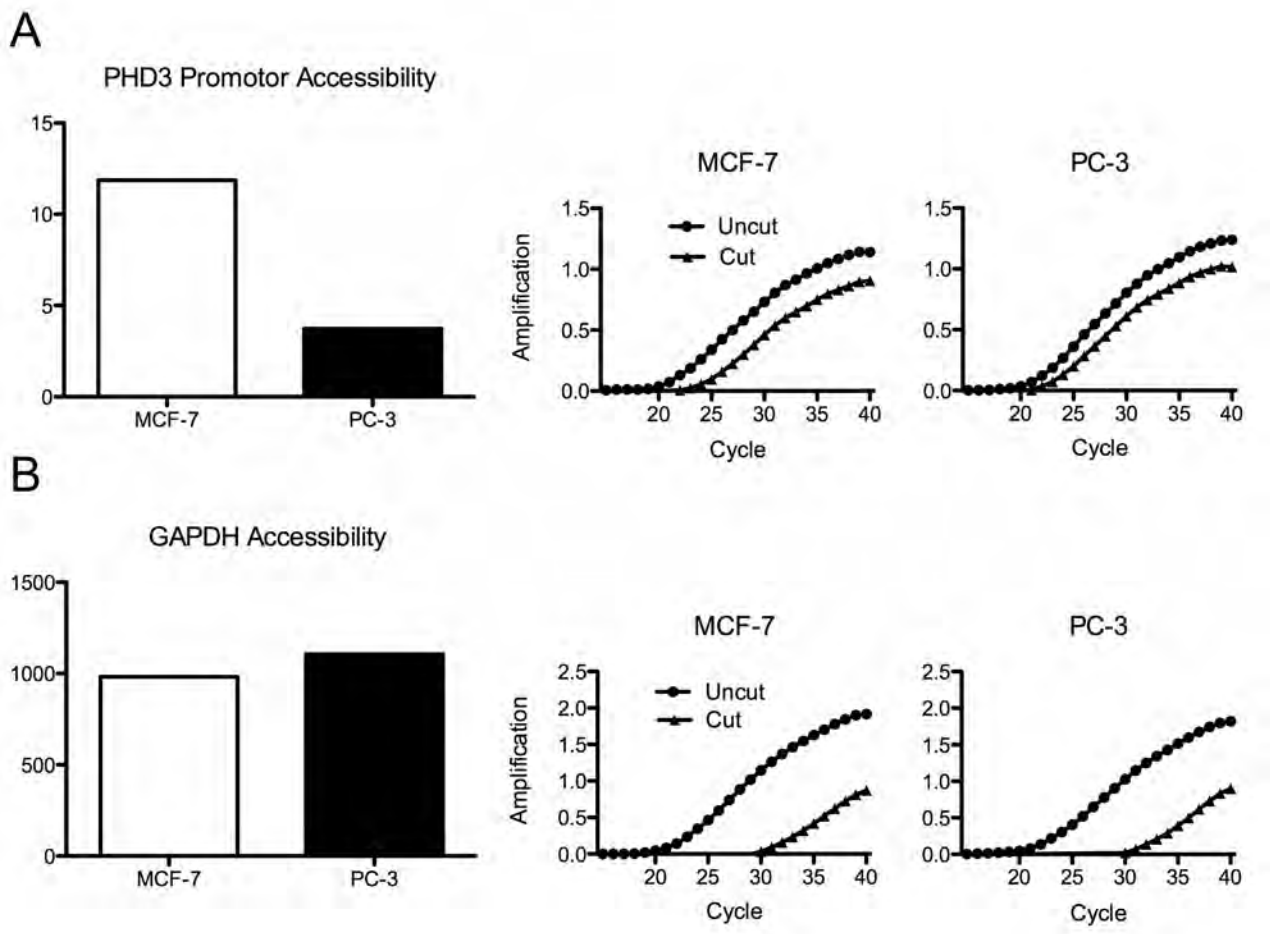

Figure 1. The methylated PHD3 gene in non-expressing cells is maintained in a less accessible state than the non-methylated PHD3 gene in expressing cells. A) Nuclei from PHD3-positive MCF-7 and PHD3-negative PC-3 carcinoma cell lines were isolated and enzymatically restricted with DNase I. Quantitative real-time PCR (right panels) was used to amplify a region also assessed for cytosine methylation. Accessibility indices (left panels) were calculated as follows: AI = 2((Ct DNase treated) – (Ct Untreated)). B) GAPDH accessibility indices were simultaneously assessed as a control for a constitutively expressed gene in both cell lines.

Appendices – Figure 2

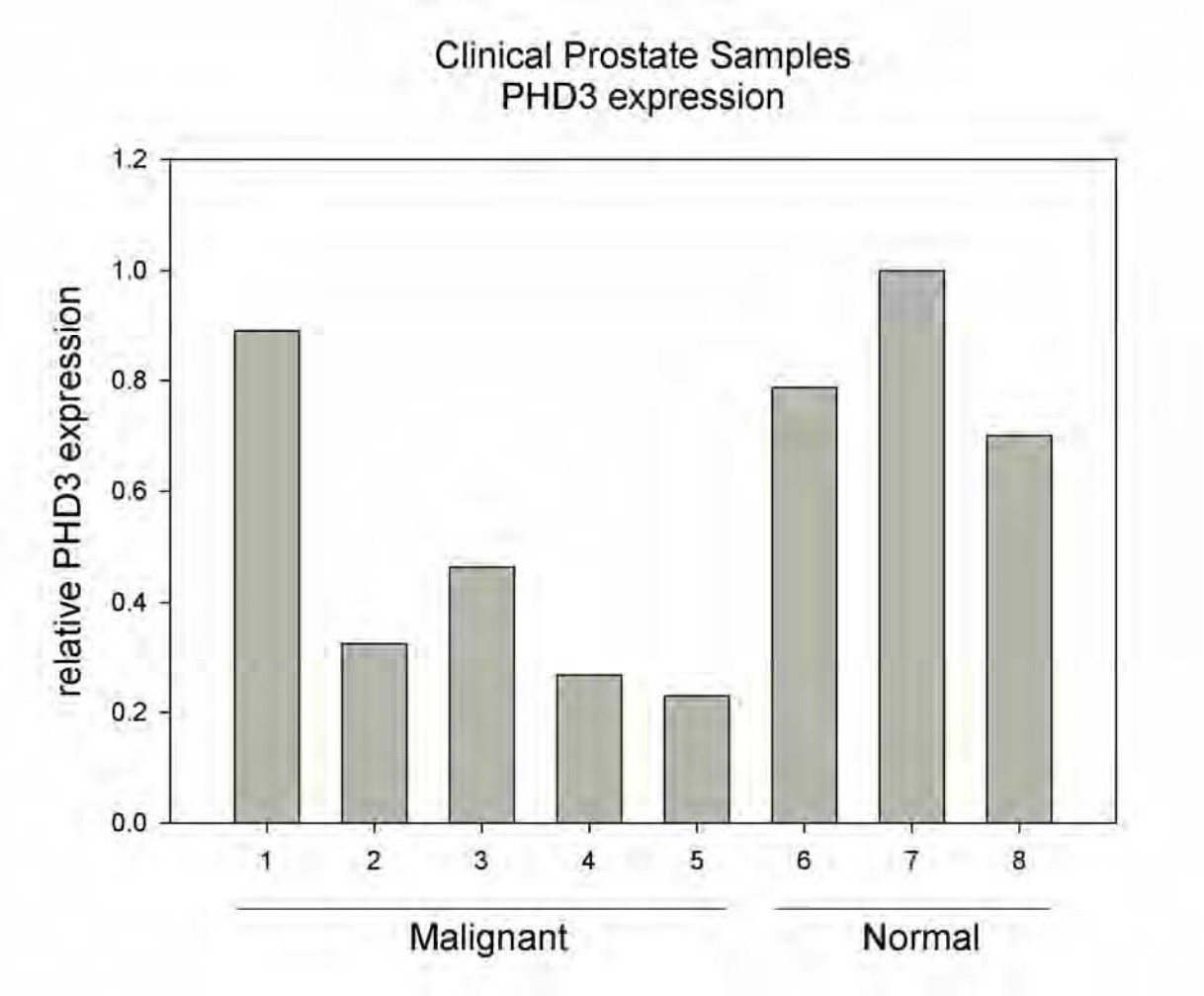

Figure 2. PHD3 mRNA is on average decreased amongst the human prostate tumors. RNA was extracted using Trizol reagent and reverse transcribed by using the ABI cDNA Archive Kit. Quantitative real-time PCR analysis was performed using primers specific to the PHD3 mRNA transcript, and data was normalized using the ΔΔCT method to the normal prostate #7 sample.

Appendices – Figure 3

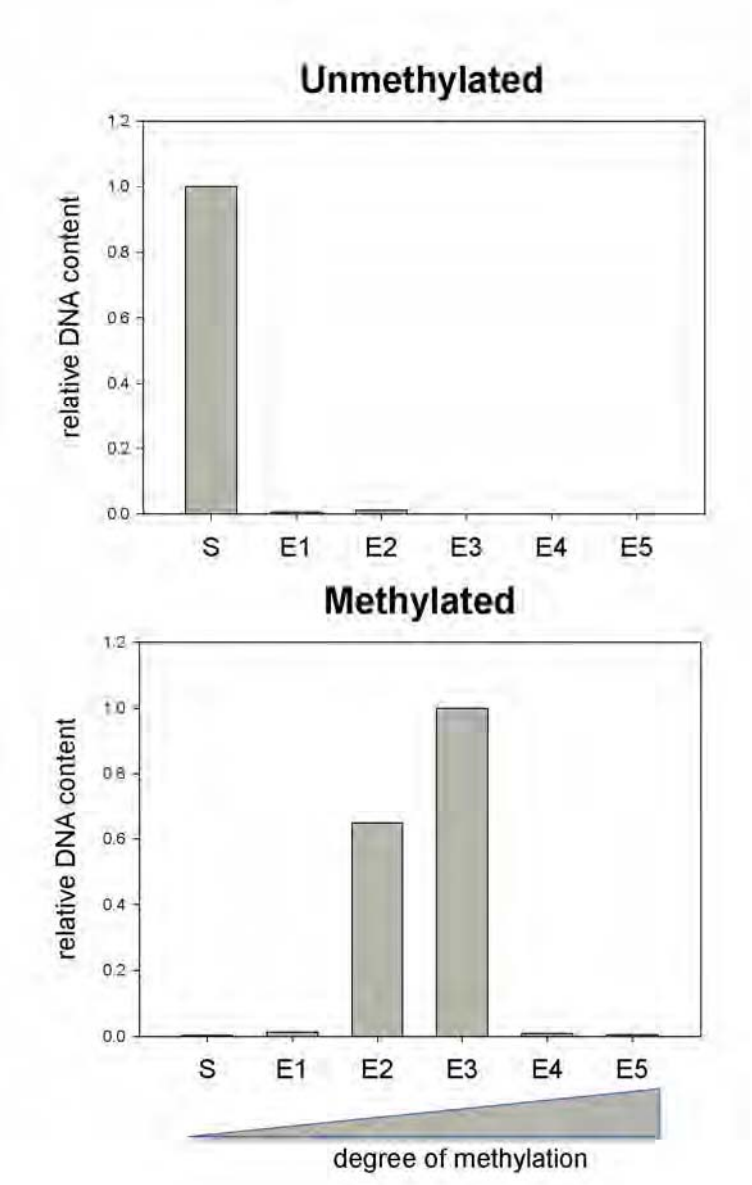

Figure 3. The MethylMiner technology appears efficient in separating methylated versus unmethylated DNA fragments. To assess the effectiveness of the assay, unmethylated (0 methylation events) and methylated (8 methylation events) control DNA were tested on the MethylMiner assay kit from Invitrogen. Briefly, DNA is sonicated to approximately 200 bp fragments, and then is exposed to specific methyl-DNA binding beads overnight. DNA not bound to the beads after this incubation remains in the supernatant (S), and is considered unmethylated. The beads are then washed with increasing salt concentrations to elute off the bound methylated DNA (E1, E2, etc). The higher the salt concentration required to remove the DNA from the bead is indicative of the increasing relative amount of methylation inherent to the sample. In this test, only the methylated control was able to be eluted off of the beads indicating the specificity and accuracy of this methodology.

Appendices – Figure 4

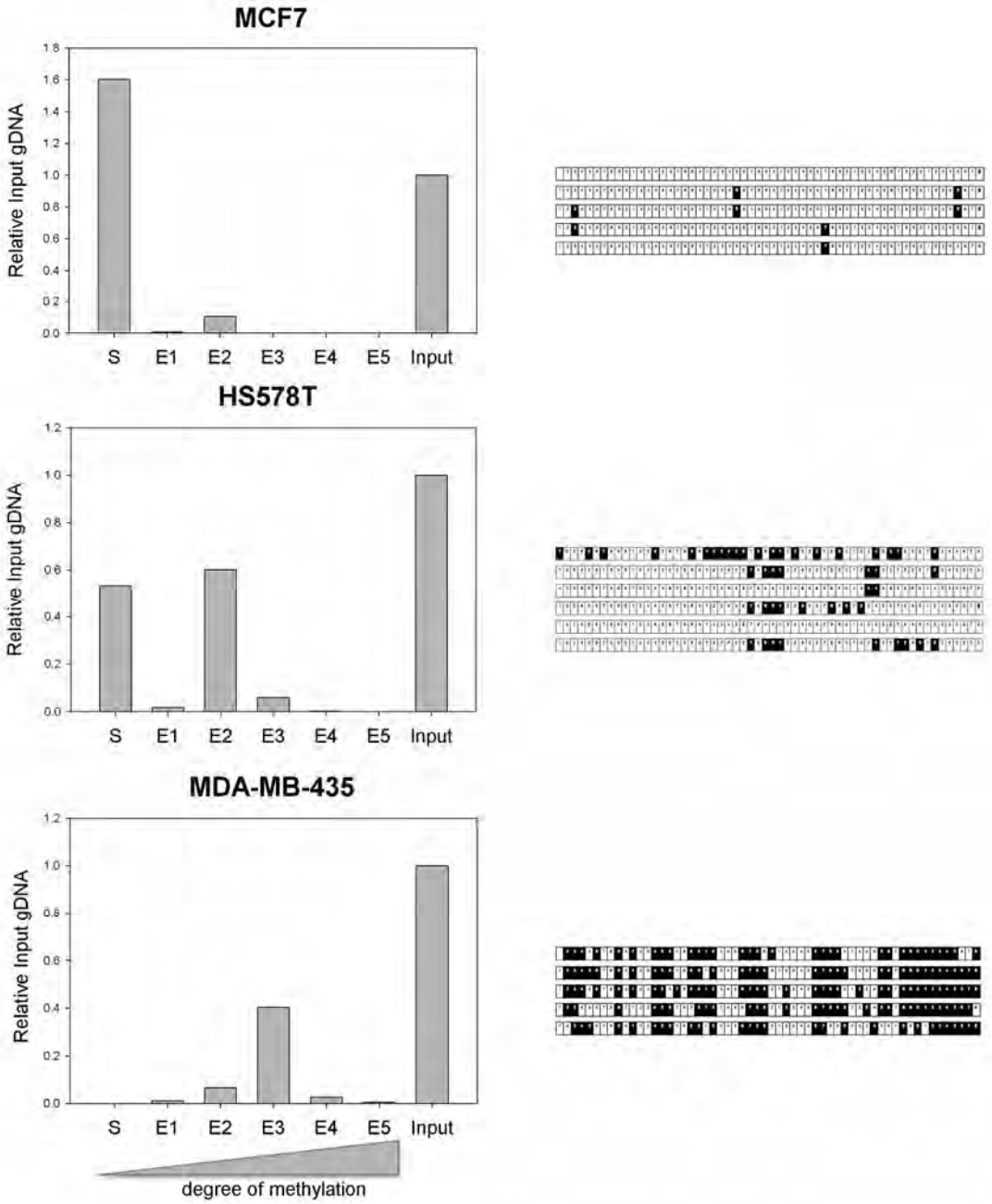

Figure 4. The MethylMiner assay correlates directly with promoter methylation analysis for PHD3. DNA extracted from the respective cell lines was tested using the assay outlined in Figure 3. After elution of DNA, quantitative real-time PCR analysis of the PHD3 promoter was performed to assess the methylation status within these cells. Results demonstrated that the low PHD3 expressing cell line (MDA-MB-435) had higher methylation, and this correlated with the DNA methylation analysis from our last report (right panel – Black squares indicate methylated CpG, White squares indicate Unmethylated CpG). The opposite was true of the high PHD3 expressing cell line MCF7, and intermediate results were found with the partial-expressing cell line HS578T.

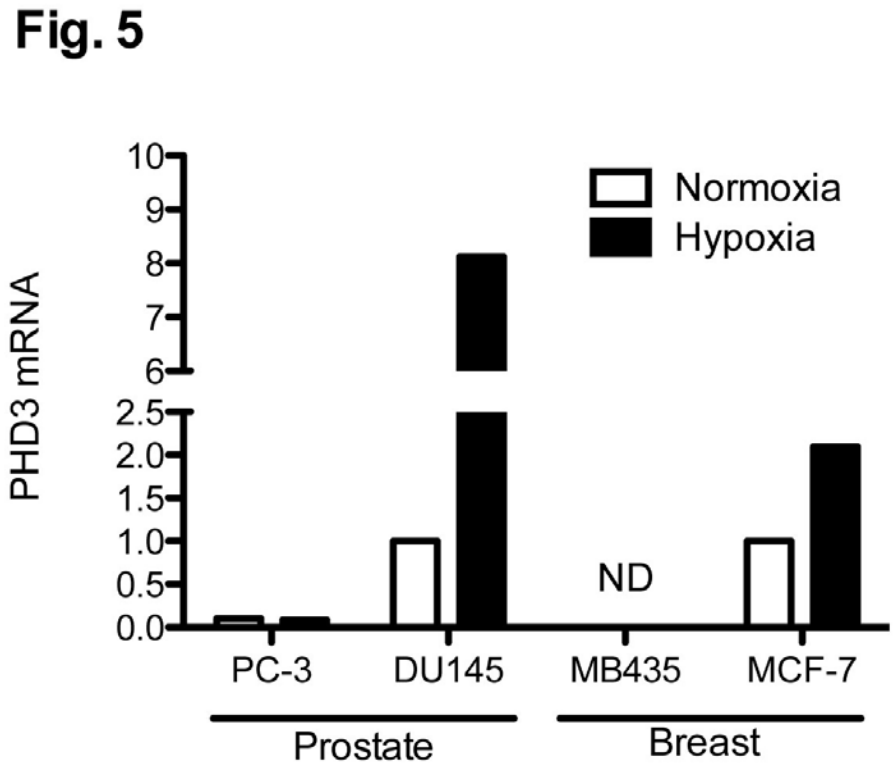

Figure 5. The methylated PHD3 gene in prostate and breast carcinoma cells is refractory to induction by hypoxia. Prostate and breast carcinoma cell lines were treated with hypoxia (1% O2) or normoxia (21% O2) for 24 hours. Total RNA was extracted and converted to cDNA by reverse transcription. Quantitative real time reverse transcription-PCR analysis of PHD3 was performed with normalization of 18S gene expressions and relative quantitation was determined by the ΔΔCt method. ND = Not detectable.

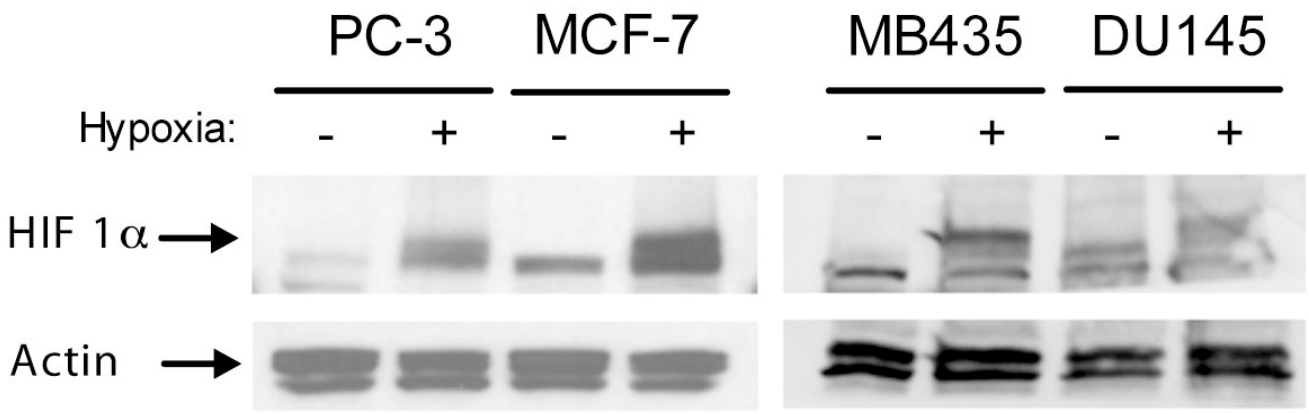

Figure 6. The HIF-1 response is not impaired in prostate and breast carcinoma cell lines that lack PHD3. Breast and prostate cancer cell lines with methylated PHD3 promoters (MB435, PC-3) and non-methylated promoters (MCF7, DU145) were subjected to hypoxia (1% O_2) or normoxia for 24 hours. Thirty µg of whole cell lysate was Western blotted for the presence of HIF-1α; actin was used as a loading control

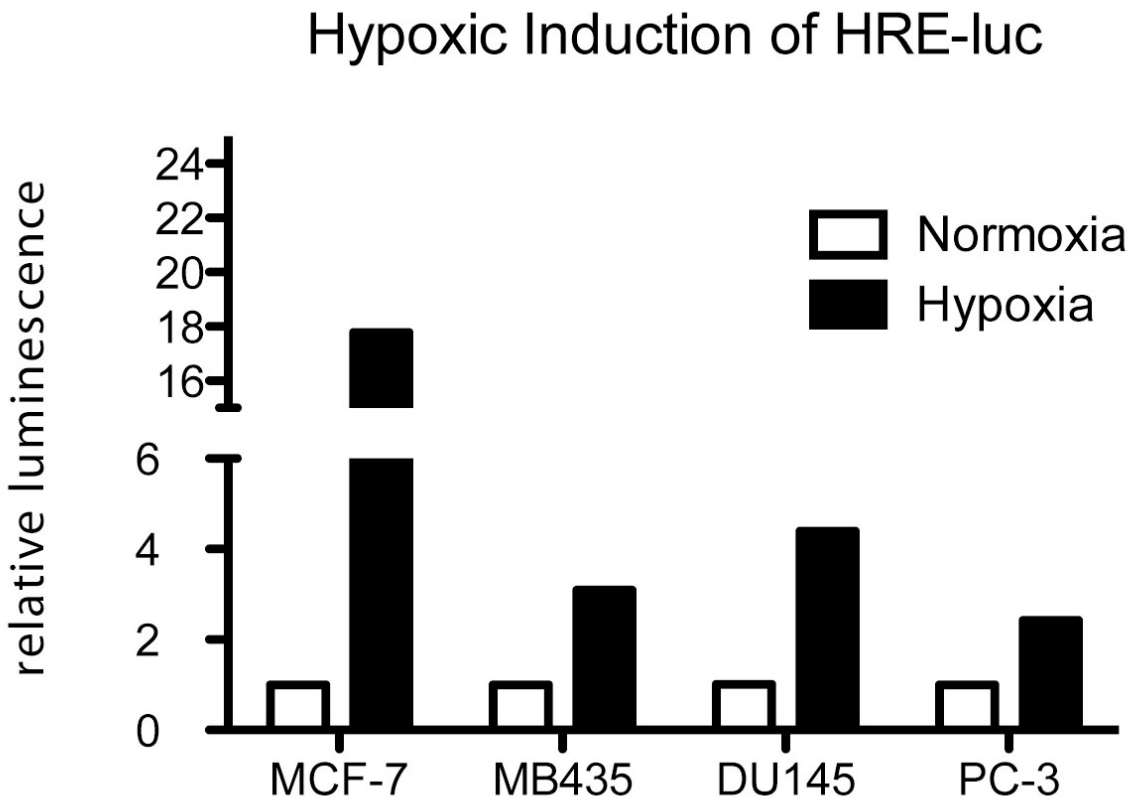

Figure 7. The HIF-1 response appears to have transactivating potential in breast and prostate cancer cell lines. Breast and prostate cancer cell lines were transfected with an HRE-luciferase reporter vector and Renilla luciferase vector and then subjected to hypoxia (1% O_2) or normoxia for 24 hours. Luciferase activities after 24 hours of hypoxia were determined and are depicted relative to luciferase activities in cells under normoxia.

REVISED STATEMENT OF WORK

Epigenetic Control of Prolyl and Asparaginyl Hydroxylases in Prostate Cancer

Adam J. Case, B.S., Predoctoral Prostate Cancer Training Award Applicant
Department of Defense Prostate Cancer Research Program

Task 1: To examine the mRNA expression and CpG methylation status of the promoters of the PHD3 enzyme in prostate carcinoma cell lines **as well as human prostate tumors** (Months 1-18)

a. Characterize the RNA expression level of the PHD3 isoform in several prostate carcinoma cell lines (DU145, PC3, LNCaP) as well as various breast carcinoma cell lines **and human prostate tumors** (Months 1-12)
b. Assess expression level changes of PHD3 in the same cell lines mentioned in (a) during exposure to hypoxia versus normoxia (Months 12-18)
c. Sodium bisulfite convert genomic DNA from each cell line and assess for methylated promoter elements along the PHD3 gene (Months 6-12)
d. Examine the global methylation status of various prostate and breast carcinoma cell lines as well as human prostate tumors using the MethylMiner technology (Months 18-36)
e. Attempt to reactivate suppressed forms of PHD3 by use of epigenetic de-methylating agents, and assess for RNA and protein expression changes (Months 12-18)

Task 2: To determine how histone modifications as well as chromatin structure function to suppress PHD3 (Months 16-24)

a. Examine chromatin structure at the level of the PHD3 promoter by way of chromatin accessibility assay (Months 16-24)
b. Attempt to reactivate the suppressed form of PHD3 by use of epigenetic histone deacetylase inhibitors, and assess for RNA and protein expression changes (Month 16-24)

Task 3: To identify relation between HIF and PHD3 expression along with elucidating feedback mechanisms of HIF upon PHD3 (Months 18-36)

a. Characterize the RNA and protein expression level of HIF-1α in prostate cell lines as well as various breast cell lines (Months 18-24)
b. Assess expression level changes of HIF-1α in the same cell lines mentioned in (a) during times of hypoxia versus normoxia (Months 18-30)
c. Obtain or generate HIF-luciferase (hypoxia response element) promoter-reporter construct (Months 24-36)
d. Transfect promoter-reporter and assess for proper HIF-1 mediated transcriptional activation to assess alterations in HIF/PHD3 feedback loop (Months 30-36)
e. Examine the role of PHD3 silencing and the transcription factor pathway NF-kB in prostate carcinoma cell lines (Months 18-36)
f. Generate and submit publication relevant to this work (Months 33-36)